

# Feeding the BT Cationic Peptides to Chickens at Hatch Reduces Cecal Colonization by *Salmonella enterica* Serovar Enteritidis and Primes Innate Immune Cell Functional Activity

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## Abstract

The BT/TAMUS 2032 (BT) cationic peptides are a group of related cationic peptides produced by a Gram-positive soil bacterium, *Brevibacillus texasporus*. Cationic amphiphilic peptides produced by host cells have been found to stimulate or prime the innate immune responses in mammals, but little information is available on the effects of bacterial-produced peptides on host immunity. We have previously shown that BT, provided as a feed additive for 4 days after hatch, significantly induced protection against extraintestinal colonization by *Salmonella enterica* serovar Enteritidis. We also found that feeding BT significantly upregulated the functional efficiency of heterophils, the avian equivalent to mammalian neutrophils. The objective of the present study was to further evaluate the effect of BT as a nonantibiotic, antibacterial compound and a stimulator of the innate immune response of young chickens. BT, provided as a feed additive at three different concentrations (12, 24, or 48 ppm) for 4 days after hatch, significantly increased protection against *Salmonella enterica* serovar Enteritidis cecal colonization in a concentration-dependent manner. We also confirmed our previous results that the functional activities of heterophils from chickens fed the BT rations were significantly upregulated. In addition, we also found that the functional activities of peripheral blood monocytes were significantly increased in a concentration-dependent manner when compared with monocytes isolated from chickens fed a control diet. This is the first report of bacterial cationic peptides providing protection against *Salmonella* cecal colonization. The significance of these data is that the orally delivered cationic peptides stimulate the innate response during the first week after hatch, normally a time of immunologic inefficiency and increased susceptibility to bacterial infections. We speculate that BT given as a feed additive during the first week after hatch could provide increased protection against a variety of bacterial pathogens because of the nonspecific nature of the innate response.

## Introduction

**T**RADITIONAL MANAGEMENT of infectious diseases in poultry has, for the large part, depended on the use of broad-spectrum antibiotics and/or vaccines. The use of antibiotics as growth promoters, concerns of antibiotic residues in meat, and increasing numbers of antibiotic-resistant isolates of *Salmonella* and *Campylobacter* causing microbial diseases in humans have resulted in a growing public outcry for the removal of antibiotics from poultry and animal feed. Ultimately, this will limit future introduction of new antibiotics for animal use. The World Health Organization is currently urging the meat-producing countries around the world to use

“environmentally and friendly” alternative methods of controlling infectious diseases (Lowenthal *et al.*, 1999). Thus, intensive research efforts are focusing on the development of novel, nonantibiotic intervention strategies including antibacterial feed additives and immunological control measures to prevent or reduce intestinal colonization of broiler flocks by these bacterial pathogens.

Recently, a novel Gram-positive bacterium, *Brevibacillus texasporus* (ATCC PTA-5854), was isolated that produces BT/TAMUS 2032 (BT), a group of structurally related cationic peptides. BT was found to be highly efficacious against a natural outbreak of colibacillosis in broiler chickens based on improved performance and reduced mortality in comparison

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to the unmedicated birds at a level (12 ppm) that was below the minimal inhibition concentration (MIC) for *Escherichia coli* (Jiang *et al.*, 2005). *In vitro*, BT displays efficient bactericidal activity against Gram-positive bacteria (MIC of 1 ppm), but a reduced efficacy against Gram-negative bacteria (MIC > 20 ppm). Interestingly, orally delivered BT seems to be completely lacking direct antibacterial activities (12 ppm) (Jiang *et al.*, 2005).

We recently reported that chickens given BT as a feed additive for the first 4 days after hatch provided protection against extraintestinal *Salmonella* infections and induced the upregulation of heterophil functional activities (Kogut *et al.*, 2007). These data were intriguing because neonatal chickens are highly susceptible to systemic dissemination of enteric *Salmonella* infections during the first week after hatch due to the inefficiency of the innate antibacterial defenses (Wells *et al.*, 1998).

*Salmonella* preferentially multiply along the mucosal surfaces of the gastrointestinal (GI) tract and exist in a stationary phase in the lumen (Poulsen *et al.*, 1995; Licht *et al.*, 1996) without being voided by gut peristalsis. Because of this ability to colonize and remain in the GI tract, contaminated birds can enter the processing plant and GI contents can contaminate both the equipment and workers' hands resulting in dramatic increases in the number of contaminated carcasses.

Therefore, the first objective of these experiments was to determine whether feeding BT to neonatal chickens can reduce cecal colonization by *Salmonella enterica* serotype Enteritidis (SE). Second, because SE cecal colonization is a longer infection model than the organ invasion model (7 days post-challenge vs. 1 day postchallenge), we were interested in evaluating the longevity of the immune modulatory effects of BT on heterophil functional activities as well as whether BT had an effect on the functional activity on another peripheral blood phagocytic cell, the monocyte.

## Materials and Methods

### MIC determination assay

SE was grown overnight at 37°C to the mid-log phase in tryptic soy broth, washed once with sterile phosphate-buffered saline (PBS, pH 7.2), and suspended to  $1 \times 10^6$  CFU/mL in PBS. Bacteria were dispensed into 96-well microtiter plates, followed by the addition of different concentrations of peptide (20, 40, 160, or 320 ppm). The microtiter plates were incubated at 41°C with shaking. The MIC was the lowest peptide concentration that produced a visually clear well. All experiments were performed in triplicate.

### Experimental animals

Experiments were conducted according to the regulations established by the U.S. Department of Agriculture animal care and use committee. Broiler chickens used in this study were obtained from a commercial breeder (Cobb 500) and were all of the same genetic background. Fertilized eggs were set in incubators (Jamesway Incubator Company, Cambridge, Ontario, Canada) and maintained at wet and dry bulb temperatures of 32.2°C and 37.8°C, respectively. After 10 days of incubation, the eggs were candled; nonfertilized and nonviable eggs were discarded. The viable eggs were returned to the incubator until day 18, when they were transferred to hatch-

ers (Natureform Hatchers, Jacksonville, FL) and maintained under the same temperature and humidity conditions until hatch. At hatch, chicks were placed in floor pens containing wood shavings, provided supplemental heat, water, and a balanced, unmedicated corn and soybean meal-based chick starter diet *ad libitum* that met or exceeded the levels of critical nutrients recommended by the National Research Council (1994). *Salmonella* were not detected in the feed or from the paper tray liners.

### Partial purification of BT

*B. texasporus* E58 cells were grown in 1 L of lysogeny broth (LB) in an air shaker at 37°C for 3 days. The culture was spun in a clinical centrifuge at 3000 g for 15 min. The supernatant was collected, and 500 g of ammonium sulfate was added and dissolved. The sample was spun in the clinical centrifuge at 3000 g for 15 min. The pellet was dissolved in 200 mL of distilled water. The solution was then boiled for 15 min and then cooled on ice. The sample was filtered with a 0.2- $\mu$ m filter (Nalgene Inc., Rochester, NY). The filtrate was mixed with 0.2 L of chloroform at room temperature for 20 min with a stir bar. The mixture was separated into two phases by centrifugation in the clinical centrifuge at 3000 g for 15 min. The organic phase was collected and dried in a vacuum evaporator.

### Premix preparation

The dried material was dissolved in ethanol, and the BT concentration was analyzed for anti-*Staphylococcus aureus* activity with a Kirby-Bauer assay (MIC = 0.8  $\mu$ g/mL). The solution was sprayed onto cornmeal and then dried. The resulting material was used as premix for the chicken studies.

### SE cecal colonization

**Bacterial challenge organism.** An isolate of SE was obtained from NVSL (Ames, IA) (ID# 9711771, PT 24). The isolate was selected for resistance to novobiocin and carbenicillin (NO-CN) and was maintained in tryptic soy broth or tryptic soy agar at 4°C. Brilliant green agar (BGA), a selective culture medium for *Salmonella*, was used to culture the resistant isolate in experimental studies and contained 100  $\mu$ g/mL CN and 25  $\mu$ g/mL NO to inhibit growth of other bacteria (BGA + NO-CN). Inoculum for challenge was prepared from 18 to 24 h tryptic soy broth + NO-CN cultures maintained at 39°C and diluted in sterile PBS (pH 7.2). A stock solution ( $1 \times 10^9$  CFU/mL) was prepared, and bacterial concentration was determined spectrophotometrically using a standard curve at a reference wavelength of  $\lambda = 625$  nm.

### Challenge experimental design

One-day-old broiler chickens were randomly distributed into four experimental groups. Groups 1–4 each contained 25 birds fed a control balanced, unmedicated corn and soybean meal-based diet that contained 0 (control), 12, 24, or 48 ppm BT, respectively, for 3 days. On the fourth day after hatch, all BT feed was removed and replaced with control diet for the remainder of the experiment. In addition, on the fourth day after hatch, all chickens were orally challenged with  $5 \times 10^6$  CFU/mL SE. Seven days after challenge (10 days after hatch), all chickens were killed by cervical dislocation and cecal contents analyzed for SE colonization.

The ceca from each chicken was removed aseptically, and the contents (0.25 g) were serially diluted to 1:100, 1:1000, or 1:10,000 and spread onto NO-CN BGA plates. The plates were incubated at 37°C for 24 h, and the number of NO-CN-resistant SE per gram of cecal contents was determined. The data from each experimental group were pooled from two separate trials for statistical analysis.

#### Statistical analysis of SE cecal colonization

The data from each experimental group were pooled from two separate trials for statistical analysis. Statistical differences between treatment groups were determined by analysis of variance ( $p < 0.05$ ). Means were further separated for significance with a pair-wise multiple comparison procedure (Tukey test,  $p < 0.01$ ) (SigmaStat User's Manual, 1994). Chi-square analysis was used to determine significant differences between groups in *Salmonella* cecal colonization rates. Differences were considered to be significant based on the 0.05 level of probability.

#### Experimental design for in vitro leukocyte assays

One-day-old broiler chickens were randomly distributed into three experimental groups. Groups 1–3 each contained 300 chickens that were fed a balanced, unmedicated corn and soybean meal-based diet that contained 0 (control), 12, or 24 ppm BT, respectively, for 3 days. On the fourth day after hatch, all BT feed were removed and replaced with control diet for the remainder of the experiment. On days 0 and 4 after the removal of the BT diets (day 0 = day 4 posthatch, day 4 = day 8 posthatch), 100 chickens from each treatment group were bled for isolation of leukocytes. On days 10 and 15 after the removal of the BT diets (day 10 = day 14 posthatch, day 15 = day 19 posthatch), 50 chickens from each treatment group were bled for isolation of leukocytes.

#### Leukocyte isolation

Chicken heterophils and peripheral blood mononuclear cells (PBMCs) were isolated from the peripheral blood as described by Kogut *et al.* (1995). Briefly, peripheral blood from approximately 100 chicks was pooled, mixed with 1% methylcellulose (1:1 v/v), and centrifuged at 25  $g$  for 15 min. The supernatant was diluted with  $\text{Ca}^{2+}$ - and  $\text{Mg}^{2+}$ -free Hank's balanced salt solution, carefully layered onto a discontinuous Histopaque gradient (specific gravity 1.077/1.119) in 50 mL conical centrifuge tubes, and centrifuged at 250  $g$  for 60 min. The PBMC layer at the 1.077/supernatant interface was collected, washed, and resuspended in RPMI-1640. The heterophils, located below the Histopaque 1.077/1.119 interface, were collected, washed, and resuspended in RPMI-1640. Heterophils and PBMCs were counted on a hemacytometer and kept on ice until use. Heterophil and PBMC preparations obtained by this method were typically >98% pure and >95% viable. Heterophils were used in the degranulation and oxidative burst assays described below, whereas PBMCs were used in oxidative burst and nitrite production assays.

Aliquots of 200  $\mu\text{L}$  of PBMCs ( $1 \times 10^7$  cells/mL) were dispensed onto a 96-well round-bottom plate and incubated at room temperature for 2 h. After incubation, nonadherent cells were removed by washing twice with the culture medium (Dulbecco's modified Eagle's medium) containing 10% chicken serum, antibiotics (100 U penicillin/mL and 100  $\mu\text{g}$

streptomycin/mL), and 1.5 mM L-glutamine. The adherent monocytes were used for the oxidative burst and nitric oxide production assays described below.

#### Assay stimulants

Opsonized *Salmonella enteritidis* (OpSE) was prepared as previously described (Ferro *et al.*, 2005). Briefly, SE ( $1 \times 10^9$  CFU/mL) were suspended in normal chicken serum (v:v [4:1]) and incubated for 60 min at 39°C on a rotary shaker. OpSE was stored at 4°C until used.

The nuclease-resistant phosphorothioate oligodeoxynucleotides (ODNs) were purchased from Integrated DNA Technologies (Coralville, IA) and further purified by ethanol precipitation. ODNs were dissolved in sterile PBS (pH 7.2) at a concentration of 1 mg/mL. The sequences of synthetic cytosine-guanosine dinucleotide (CpG)-ODN #17 used in the present study were GTC GTT GTC GTT GTC GTT as described previously (He *et al.*, 2005).

#### Degranulation assay

Degranulation was detected by quantifying the amount of  $\beta$ -D glucuronidase activity in the culture medium following stimulation of the heterophils with normal chicken serum OpSE. Heterophils ( $8 \times 10^6$  cells/mL) were incubated with OpSE ( $8 \times 10^8$  CFU/mL) for 1 h on a rocker platform at 39°C in a 5%  $\text{CO}_2$  incubator. The reaction was stopped by transferring the tubes containing the cells to an ice bath for 5–10 min. The cells were then centrifuged at 250  $g$  for 10 min at 4°C. The supernatants were then removed and used for the assay. A 25  $\mu\text{L}$  aliquot of each supernatant was added to quadruplicate wells in a nontreated, black CoStar flat-bottom ELISA plate and incubated with 50  $\mu\text{L}$  of freshly prepared substrate (10 mM 4-methylumbelliferyl- $\beta$ -D-glucuronide, 0.1% Triton X-100 in 0.1 M sodium acetate buffer) for 4 h at 41°C. The reaction was stopped by adding 200  $\mu\text{L}$  of stop solution (0.05 M glycine and 5 mM EDTA; pH 10.4) to each well. Liberated 4-methylumbelliferone was measured fluorimetrically (excitation wavelength of 355 nm and an emission wavelength of 460 nm) with a fmax fluorescence microplate reader (Molecular Devices, Sunnyvale, CA). These values were converted to micromoles of 4-methylumbelliferone generated using a standard curve of known concentrations. At least three replicates were conducted for each assay with the heterophils from each pool of chicken.

#### Oxidative burst assay

Production of an oxidative burst by phorbol myristate acetate-stimulated chicken heterophils and monocytes was measured by oxidation of 2',7'-dichlorofluorescein-diacetate to fluorescent dichlorofluorescein as described previously (He *et al.*, 2003) with modification. One milliliter of chicken heterophils ( $8 \times 10^6$  cells/mL) was added to 2-mL microcentrifuge tubes and then incubated with phorbol myristate acetate (1.62  $\mu\text{M}$ ) and dichlorofluorescein-diacetate (10  $\mu\text{g}$ /mL in final concentration) for 1 h at 41°C. The aliquots of cell cultures (150  $\mu\text{L}$ ) were then dispensed to a black 96-well plate, and the fluorescence was measured using a GENios Plus Fluorescence Microplate Reader (TECAN US, Research Triangle Park, NC) at 485 nm excitation and 530 nm emission wavelengths. The relative fluorescent units were recorded after 60 min. At least

three replicates were conducted for each assay with the heterophils from each pool of chicken.

#### Nitrite assay

The adherent monocytes in 200  $\mu$ L culture medium per well were stimulated with CpG-ODN #17 for 48 h at 41°C in a 5% CO<sub>2</sub> and 95% humidity incubator. Nitrite produced by activated monocytes was measured by the Greiss assay (Green *et al.*, 1982). Briefly, an aliquot of 100  $\mu$ L culture supernatant from each well was transferred to a new 96-well plate (flat bottom) and combined with 50  $\mu$ L of 1% sulfanilamide and 50  $\mu$ L of 0.1% naphthylethylenediamine (both were prepared in 2.5% phosphoric acid solution). After 10 min incubation at room temperature, the nitrite concentration was determined by measuring optical density (OD 595) of each well using a SPECTRA MAX microplate reader (Molecular Devices). Sodium nitrite (Sigma Chemical, St. Louis, MO) was used as a standard to determine nitrite concentrations in the cell-free medium. At least three replicates were conducted for each assay with the heterophils from each pool of chicken.

#### Statistical analysis of in vitro cell functional assays

The anticoagulated blood from either 50 (on days 10 and 15 after BT removal) or 100 (on days 0 and 4 after BT removal) chickens per treatment group described was pooled, and the heterophils and monocytes were isolated from each treatment group as described above. The data from these three repeated experiments were pooled for presentation and statistical analysis. Statistical differences between treatment groups were determined by analysis of variance ( $p < 0.05$ ). Means were further separated for significance with a pair-wise multiple comparison procedure (Tukey test,  $p < 0.05$ ) (SigmaStat User's Manual, 1994). Differences between the nonstimulated heterophils or monocytes and the stimulated cells were determined by analysis of variance. Sig-

nificant differences were further separated using Duncan's multiple range test.

## Results

#### MIC determination

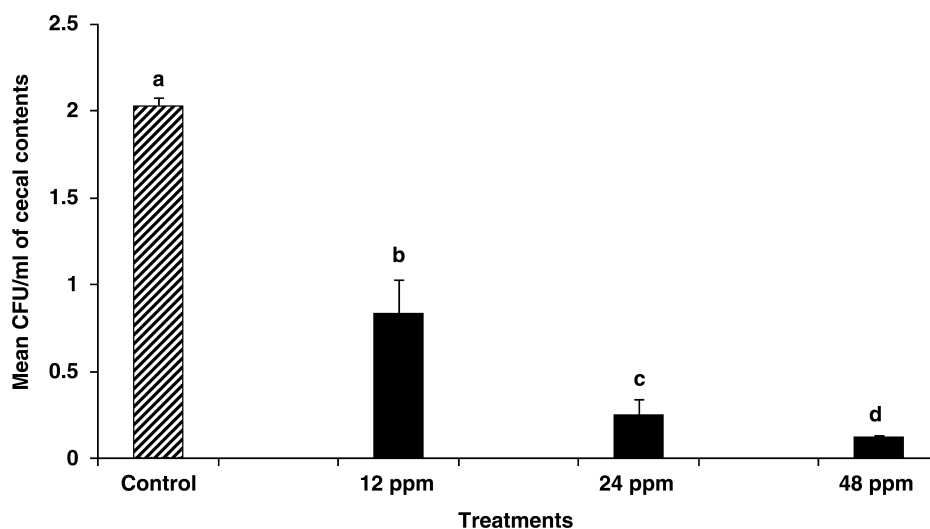
The BT MIC for the SE serovar used in these experiments was determined experimentally to  $>160$  ppm. Based on this result, we selected BT concentrations well below the MIC (12, 24, and 48 ppm) for the *in vivo* studies.

#### SE cecal colonization

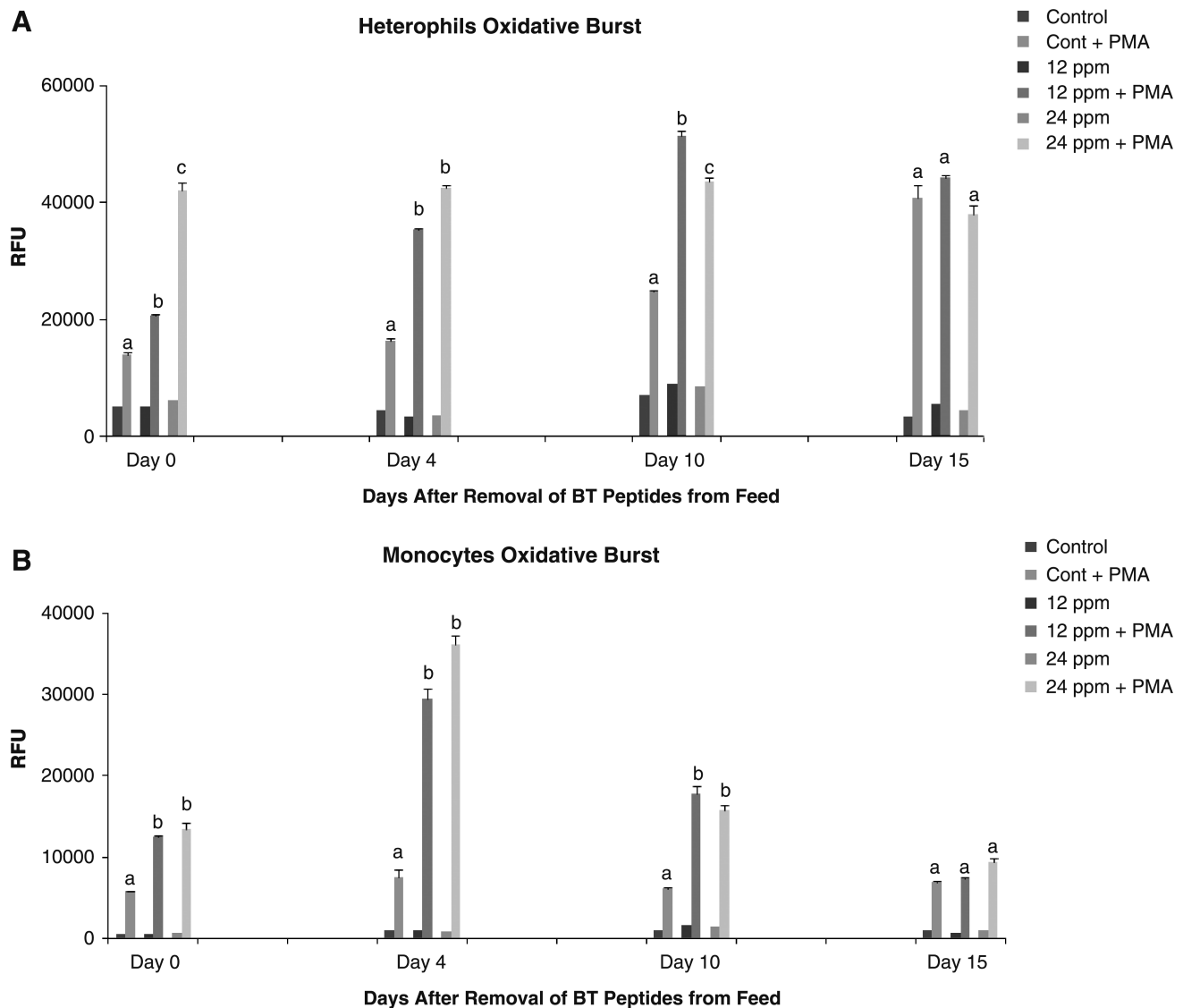
The numbers of *S. enteritidis* (CFU/mL) in the cecal contents of chickens in all BT-fed groups in both experiments were significantly less than in the control diet-fed group (Fig. 1). Similarly, the percentage of *S. enteritidis* cecal culture-positive chickens in the BT-fed groups was significantly fewer than the control diet-fed chickens (data not shown).

#### Leukocyte functional assays

**Heterophils.** We have previously shown that feeding the BT peptides to chickens for the first 4 days after hatch had a priming action for heterophil degranulation, and oxidative burst 1 day after the removal of BT from the feed (Kogut *et al.*, 2007). In the present experiment, we investigated the longevity of this priming action following removal of the BT peptide from the feed. The priming activity induced by feeding BT was found to last for up to 10 days after the BT supplemented was removed when compared with the heterophils from chickens fed the control diet (Fig. 2A). By 15 days after BT feed removal, there were no differences in the oxidative burst activity by heterophils isolated from either the control or BT-fed diets. Peak priming activity induced by the BT diet appeared to occur by 10 days after BT feed removal.



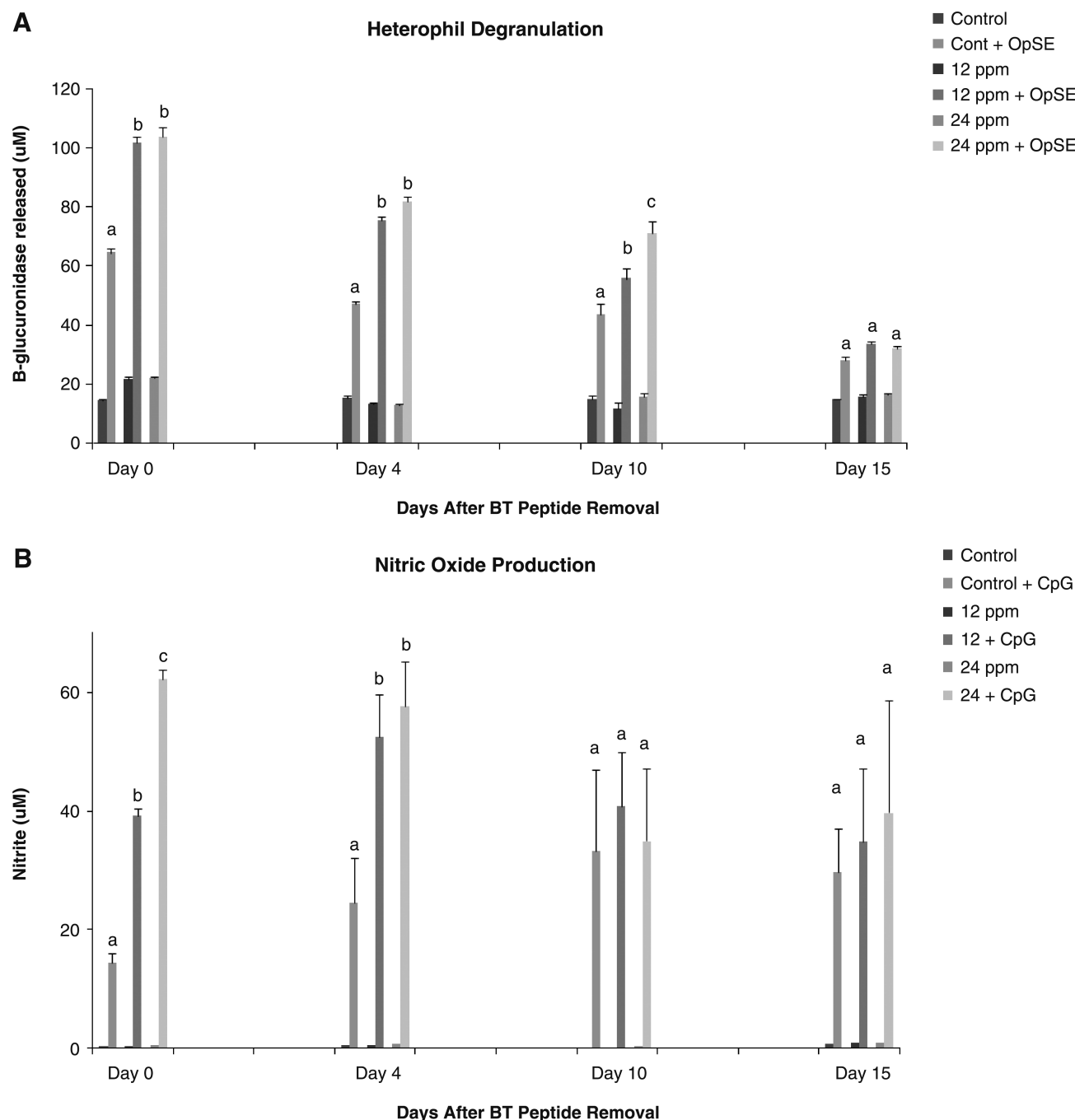
**FIG. 1.** One-day-old broiler chickens were randomly distributed into four experimental groups. Groups 1–4 each contained 25 birds fed a control balanced, unmedicated corn and soybean meal-based diet that contained 0 (control), 12, 24, or 48 ppm BT/TAMUS 2032 (BT), respectively, for 3 days. On the fourth day after hatch, all BT feed were removed and replaced with control diet for the remainder of the experiment and all chickens were orally challenged with  $5 \times 10^6$  CFU/mL *Salmonella enterica* serovar Enteritidis (SE). Seven days after challenge (10 days after hatch) all chickens were killed and cecal contents analyzed for SE colonization. Different superscript letters indicate the significant difference ( $p < 0.05$ ) in SE cecal colonization.



**FIG. 2.** (A) Heterophils isolated from chickens fed a control diet were compared with heterophils isolated from chickens fed the BT-supplemented diet (12 or 24 ppm). Reactions contained  $8 \times 10^6$  heterophils per well,  $10 \mu\text{g}$  dichlorofluorescein-diacetate/mL, and  $1 \mu\text{g/mL}$  phorbol myristate acetate. Plates were incubated for 1 h at  $39^\circ\text{C}$  and the relative fluorescent units (RFU) were measured. Data represent the mean  $\pm$  standard error of mean (SEM) of three independent assays. Functional differences were determined by analysis of variance (ANOVA). Significant differences were further separated by Duncan's multiple range test. Different superscript letters indicate the significant difference ( $p < 0.05$ ) of RFU between the heterophils fed the cationic peptide ration when compared with heterophils from chickens fed the control ration. (B) Monocytes isolated from chickens fed a control diet were compared with monocytes isolated from chickens fed the BT-supplemented diet (12 or 24 ppm). Reactions contained  $8 \times 10^5$  monocytes per well,  $10 \mu\text{g}$  dichlorofluorescein-diacetate/mL, and  $1 \mu\text{g/mL}$  phorbol myristate acetate. Plates were incubated for 1 h at  $39^\circ\text{C}$ , and the RFU were measured. Data represent the mean  $\pm$  SEM of three independent assays. Functional differences were determined by ANOVA. Significant differences were further separated by Duncan's multiple range test. Different superscript letters indicate the significant difference ( $p < 0.05$ ) of RFU between the monocytes fed the cationic peptide ration when compared with monocytes from chickens fed the control ration.

As was observed with heterophil oxidative burst activity, priming of heterophil degranulation induced by the BT-supplemented diet was found to be significantly different from the heterophils from the control diet-fed birds through 10 days after BT feed removal (Fig. 3A). However, optimal priming appeared to occur within the first few days after the BT feed removal.

**Monocytes.** Feeding BT to chickens for the first 4 days after hatch also primed peripheral blood monocytes for a significant increase in oxidative burst when compared with the monocytes from chickens fed the control diet (Fig. 2B). The BT-induced priming activity was found to last for up to 10 days after the BT supplemented was removed from the diet with a peak of activity found 4 days after the removal of the



**FIG. 3.** (A) Heterophils isolated from chickens fed a control diet were compared with heterophils isolated from chickens fed the BT-supplemented diet (12 or 24 ppm). Degranulation by heterophils was stimulated by opsonized *Salmonella enteritidis* (OpSE). Heterophils ( $8 \times 10^6$ /mL) were incubated with OpSE ( $1 \times 10^8$  CFU/mL) for 1 h at  $39^\circ\text{C}$ . Data represent the mean  $\pm$  SEM of three independent assays. Functional differences were determined by ANOVA. Significant differences were further separated by Duncan's multiple range test. Different lower case letters indicate the significant difference ( $p < 0.05$ ) in  $\beta$ -D-glucuronidase release between the OpSE-stimulated heterophils fed the cationic peptide ration when compared with heterophils from chickens fed the control ration. (B) Comparison of nitric oxide induction in chicken monocytes by cytosine-guanosine oligodeoxynucleotide (CpG-ODN) #17. Nitrite was measured in the supernatant of monocytes (adherent cells from  $2 \times 10^6$  peripheral blood mononuclear cells per well) stimulated with CpG-ODN #17 at concentrations of 0.1, 1, and 10 mg/mL for 48 h. Different lower case letters indicate the significant difference ( $p < 0.05$ ) in nitric oxide release between the CpG-ODN-stimulated monocytes fed the cationic peptide ration when compared with monocytes from chickens fed the control ration.

TABLE 1. CHARACTERISTICS OF INNATE IMMUNITY THAT CAN BE MANIPULATED

- |   |
|---|
| 1. Rapid action: limit duration<br>Does not increase with repeated exposure |
| 2. Limited specificity<br>Does not discriminate between pathogens           |
| 3. Ability to be augmented  |

BT-supplemented feed (Fig. 2B). By 15 days after BT feed removal, there were no differences in the oxidative burst activity by heterophils isolated from chickens fed either the control or BT-fed diets.

As was observed with monocyte oxidative burst activity, priming of monocyte nitric oxide production induced by the BT-supplemented diet was found to be significantly different from the monocytes from the birds fed the control diet through the first 4 days after BT feed removal (Fig. 3B). By 10 days after BT feed removal, there were no differences in the nitric oxide activity by monocytes isolated from chickens fed either the control or BT-fed diets.

## Discussion

Both viral and bacterial diseases remain a threat to the poultry industry, and countermeasures to prevent and control them are needed due to food safety issues and production losses. With the continued threat of exotic and emerging diseases and concern over the use of antibiotics in animal production, there is a serious and urgent need to find safe and practical alternatives to prevent and/or control pathogens. The design of new immunological interventions or therapeutic antimicrobials to reduce microbial pathogens in poultry is now, more than ever, required. Based on the data provided herein and in previous experiments (Jiang *et al.*, 2005; Kogut *et al.*, 2007), the use of BT peptides as a feed additive for poultry may be an important component of an on-farm program for the control of food safety pathogens.

There are three characteristics of innate immune responses (Table 1) that we have begun exploiting for targeting for the design of immunomodulatory and/or antimicrobial compounds for protection or treatment of infections. Since the innate immune response is not pathogen specific, the ability to stimulate the response in birds is a promising approach of increasing resistance to a variety of pathogens. The one characteristic of the innate response that we have exploited is its ability to be modulated during the first week after hatch (Kogut *et al.*, 1995; He *et al.*, 2005; Genovese *et al.*, 2007). We have shown that the stimulated response results in an increased resistance to *Salmonella* infections with concomitant increases in heterophil functional activity. We have shown that using different natural molecules, toll-like receptor agonists, increased the functional immune responses of chicken heterophils (He *et al.*, 2005; Genovese *et al.*, 2007). Further, we found that this stimulation was self-limiting, lasting 3–5 days after administration of the agonist. These results indicate that the innate immune responses can be augmented and that natural products can potentially be used as antimicrobial compounds.

In the present experiments we provide further proof that BT, a group of related cation amphipathic peptides of 13 amino acids isolated from a Gram-positive soil bacterium,

possesses antibacterial activity due to the immunomodulation of peripheral blood leukocyte functional activities when supplied as a feed additive to young chickens. Providing BT as a feed additive reduces SE cecal colonization by 59% (12 ppm) to 94% (48 ppm). This protection is not the result of direct antibacterial activity of the BT on the SE since the concentrations (12, 24, and 48 ppm) used here are below the MIC (>160 ppm) for SE. This lack of direct antibacterial activity confirms the results of our earlier experiments (Jiang *et al.*, 2005; Kogut *et al.*, 2007). Collectively, the results from a series of experiments strongly suggest that the ability to deliver BT cationic peptides in the feed has promising potential as a nonantibiotic, antibacterial agent for reducing pathogenic bacterial colonization of the poultry intestine. Further studies are ongoing to evaluate the effect of feeding BT peptides on intestinal colonization by other foodborne pathogens such as *Campylobacter* and *Clostridium perfringens*.

Further, we have previously shown (Kogut *et al.*, 2007) and have confirmed in the present study that feeding BT peptides to young chickens for 4 days immediately after hatch results in the priming of heterophil functional responses to different stimuli (Fig. 2A, B). Additionally, in the present study, we show for the first time that feeding BT peptides also primes monocyte functional responses (Fig. 3A, B). However, the priming effect of BT appears to be more dramatic and longer lasting in heterophils than in monocytes. Peak priming in monocytes was found in the first 4 days after the removal of the BT feed, whereas the priming activity in heterophils peaked at 10 days after BT removal. The mechanisms of this differential priming effects between the different cell populations are unknown. However, the innate immune cell priming described here is different than that found with the mammalian host-defense peptide (IDR-1) that affects monocytes and macrophages, but not neutrophils (Scott *et al.*, 2007).

Cationic antimicrobial peptides encompass a large group of small peptides that are widely distributed in most forms of life from microbes to invertebrates to plants to humans (Brogden *et al.*, 2003; Vizioli and Salzat, 2002; Linde *et al.*, 2008). Functionally, these peptides modulate the immune response and/or direct antimicrobial activity (Ganz, 2003; Bowdish *et al.*, 2005; Hancock and Sahl, 2006; Mookherjee and Hancock, 2007; Scott *et al.*, 2007; Linde *et al.*, 2008). In mammals, most of these cationic peptides have little direct antimicrobial activity, but do enhance the innate immune responses without the harmful inflammatory responses (Finlay and Hancock, 2004; Bowdish *et al.*, 2005; Mookherjee and Hancock, 2007; Scott *et al.*, 2007). The BT peptides used in the present studies have similar characteristics to these “host-defense peptides” in contrast with the “antimicrobial peptides” that have direct antibiotic-like activity on pathogens (Hancock and Sahl, 2006). In addition, preliminary experiments have shown a growth-promoting effect (improved weight gains and feed conversion) in broiler chickens fed BT peptides continuously for a 42-day growout (Jiang, unpublished results). To date, we have observed no obvious toxic effects normally associated with an uncontrolled inflammatory response in chickens provided BT peptides as a feed additive. Thus, because of its low MIC, but strong innate immune modulatory activities, we suggest that BT peptide may be considered as a host-defense peptide when provided as a feed additive to chickens.

In summary, BT, provided as a feed additive at three different concentrations (12, 24, or 48 ppm) for 4 days after hatch,

significantly increased protection against SE cecal colonization in a concentration-dependent manner. We also confirmed our previous results that the functional activities of heterophils from chickens fed the BT rations were significantly up-regulated. In addition, we also found that the functional activities of peripheral blood monocytes were significantly increased in a concentration-dependent manner when compared with monocytes isolated from chickens fed a control diet. Thus, cationic peptides, provided as a feed additive, stimulated the innate response at the time of immunologic inefficiency and increased susceptibility to bacterial infections (first week after hatch). Because of the nonspecific nature of the innate response, we speculate that BT given as a feed additive during the first week after hatch could provide increased protection against a variety of bacterial pathogens.

### Disclosure Statement

No competing financial interests exist.

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